AD_____

GRANT NO: DAMD17-93-J-3027

TITLE: SKELETAL MUSCLE ISCHEMIA AND HEAT SHOCK PROTEINS

PRINCIPAL INVESTIGATOR: Wolfgang H. Dillman, M.D.

CONTRACTING
ORGANIZATION: The Regents of the University of California
University of California, San Diego
9500 Gilman Drive
La Jolla, California 92093-0934

REPORT DATE: July 26, 1994

TYPE OF REPORT: Annual Report

PREPARED FOR: U.S. Army Medical Research and Materiel Command,
Fort Detrick
Frederick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 26, 1994	Annual (7/1/93 - 6/30/94)

4. TITLE AND SUBTITLE
SKELETAL MUSCLE ISCHEMIA AND HEAT SHOCK PROTEINS

5. FUNDING NUMBERS
DAMD17-93-J-3027

6. AUTHOR(S)
Wolfgang H. Dillmann, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
THE REGENTS OF THE UNIV OF CALIF
UNIV OF CALIF, SAN DIEGO
9500 Gilman Drive
La Jolla, CA 92093-0934

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick
Frederick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 words)

Blood loss resulting in decreased organ perfusion and subsequent ischemic injury of cardiac and skeletal muscle presents a significant problem for the soldier in combat. Recent findings have indicated that different forms of noxious stress including exposure to increased temperature, noxious chemical agents, and ischemia lead to increased expression of heat shock proteins (HSP) which have a protective effect against injury induced by noxious stimuli. We will determine in this proposal if a rat skeletal muscle derived permanent cell line, L6 cells, expressing increased amounts of HSP70 will show protection against damaged induced by simulated ischemia. To generate L6 cells which permanently overexpress the inducible HSP70 proteins, cells will be transfected with a neomycin resistance gene and the inducible HSP70 gene. Stable lines will be selected by growing L6 cells in the presence of neomycin. Cells which have the neomycin resistance gene and the HSP70 gene incorporated into their DNA will survive. Such stably transfected L6 cell lines will then be exposed to simulated ischemia consisting of hypoxia, absence of glucose, low tonicity, and resultant ischemic damage will be determined by quantitating cell viability measured in colony formation assays, the inhibition of protein synthesis and the release of cytoplasmic enzymes like creatine kinase. These studies will determine if inducible HSP70 exerts a protective effect against ischemia mediated muscle injury. Demonstrating a protective effect of HSP70 protein will make it a useful agent to reduce ischemic muscle damage in soldiers exposed to muscle injury in combat.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Noxious stress, ischemia, protein against noxious stress, HSP70, protein folding	9
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the US Army.

N/A Where copyrighted material is quoted, permission has been obtained to use such material.

N/A Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

YES Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

YES In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

N/A For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

YES In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

YES In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

YES In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Accesion For	
NTIS CRA&I	☒
DTIC TAB	☐
Unannounced	☐
Justification	
By	
Distribution /	
Availability Codes	
Dist	Avail and / or Special
A-1	

W. D. Ullmann 7-27-94

PI - Signature Date

TABLE OF CONTENTS

INTRODUCTION

This research effort is directed at subobjective 2 as defined in the recent shock trauma mediators review memo from the U.S. Army Institute of Surgical Research. Specifically, we aim at the prevention of secondary damage after hemorrhage by temporizing fundamental physiological or biochemical processes leading to cell death and organ failure. For this purpose, increased expression of the inducible heat shock protein 70 (HSP70i) will be employed. HSP70i works as a chaperone attaching to short hydrophobic peptide sequences as they are exposed in ischemic cells in which proteins undergo denaturation. By the association of HSP70 with such protein sequences, further protein aggregation and cell damage is prevented. This leads to a faster recovery of cell function after the ischemic or hemorrhagic episode is reversed. Our results which are further summarized in the body indicate that overexpression of HSP70 in transgenic muscle-type cell lines lead to increased protection of such cells against ischemic damage. In addition, recent work in transgenic mice in which the HSP70 protein was overexpressed in heart, brain and skeletal muscle show, especially for studies in the heart, that the ischemic myocardium shows enhanced protection against ischemia related injury. In discussing these studies with Col. Smallridge, M.D., specific areas of interest at Walter Reed Army Hospital were identified. They relate to the prevention of ischemic hemorrhagic injury resulting in brain, kidney and gut. For this purpose, additional transgenic animals are bred leading to increased expression of heat shock proteins in these organs.

BODY

In recent studies which are published in the Journal of Clinical Investigation (1994;93:759-767, copy attached), we used the myocytic cell line H9c2 cells. These cells were initially derived from the fetal myocardium of the rat. These cells grow initially as myoblasts then fuse into myotubes. The cells were engineered using a stable transfection protocol to overexpress the human HSP70i gene which was kindly provided by Dr. Richard Morimoto using a neomycin selection approach. Exposing these cells to simulated ischemia which include hypoxia (oxygen tension of less than 0.04%) and the absence of glucose in the medium. After the episode of simulated ischemia, the incubation chamber is opened and cells were re-exposed to oxygen. In these cells, colony survival was determined by standard methods. In addition, we determined LDH release and the intactness of mitochondria using the vital dye rhodamine 123. As indicated in this paper, cells overexpressing the human inducible HSP70 showed a significant survival advantage after simulated ischemia using the colony survival assay in addition a much lower amount of LDH was released from these cells and mitochondria show less damage. The studies, therefore, show that excess expression of HSP70 leads to increased protection against an ischemic damage.

To confirm the increased protection which is mediated by HSP70 in cell culture in an *in vivo* system, we took the following approach. We produced transgenic mice which overexpress a HSP70 transgene. In the transgene, we used the human cytomegalovirus enhancer linked to a chicken β-actin promoter and the chicken β-actin intron. In the multiple cloning site following the chicken β-actin intron, we placed a full length cDNA coding for the rat HSP70i. Several lines of transgenic mice were obtained expressing the heat shock proteins at a very high level (Figure 1). A Western blot which is attached shows that high

levels of the rat HSP70i transgene is expressed in heart, skeletal muscle, and brain in the heterozygous lines of these mice. We are currently breeding homozygous lines in which expression in kidney and gut will be evaluated. Hearts were obtained from control mice and transgene positive mice and an isolated Langendorf heart preparation was established. These hearts were then submitted to global ischemia for 20 minutes and then reperfusion was started. At different time points after reperfusion, the contractile performance of these hearts were determined by attaching a force transducer to the apex of the mouse hearts. In addition, infarct size was quantitated using a tetrozolium based technique. Our results which are currently submitted for publication to the Journal of Clinical Investigation indicate that in the hearts obtained from transgenic mice overexpressing the heat shock protein functional recovery was improved by 30% in comparison to control hearts. In addition, infarct size was reduced by 40%. Furthermore, the liberation of creatine kinase was markedly diminished. These results, therefore, were similar to those described in cell lines above that expression of the inducible heat shock protein has a marked protective effect against ischemia. In addition to the studies described above, we have cloned the inducible HSP70 into an adenovirus vector (Figure 2). We are currently in the process of preparing for studies in rabbits. In these studies, the adenovirus coding for HSP70 will be infused into the rabbit coronary and infarct sizing will be obtained in rabbits infused with the adenovirus positive for the HSP70 transgene and adenovirus coding only for β-galactosidase. This adenovirus will be available to Dr. Smallridge's group in the future for specific studies which are of their interest.

CONCLUSION

In summary, we could show in a stable striated muscle cell line that expression of the human inducible HSP70 exerts a protective effect against simulated ischemic damage under cell culture conditions. In addition, we could demonstrate that a protective effect against ischemic damage also occurs under *in vivo* conditions in mice in which the rat inducible heat shock protein is expressed in the heart. These mice also express excess amounts of HSPs in brain and skeletal muscle and will be available for investigators at the Walter Reed Army Hospital to determine if a protective effect can be elicited in these organs. Furthermore, we have constructed an adenovirus expressing the inducible heat shock protein. This adenovirus will be available to determine in cell lines of interest to investigators at the Army Hospital like the 431 cell line and GH3 cell lines if a protective effect of heat shock proteins can also be elicited in these cells. We, therefore, think we have been highly productive in our research effort demonstrating that increased expression of heat shock proteins can have a protective effect against ischemic damage. By using adenovirus or similar particle in the future it will be possible to harness the protective effects of specific heat shock proteins using a gene therapy based approach.

REFERENCES

1. Mestril R, Chi S-H, Sayen MR, Dillmann WH: Isolation of a novel inducible rat heat shock protein (HSP70) gene and its expression during ischaemia/hypoxia and heat shock. Biochem J 1994;298:561-569.

2. Mestril R, Chi S-H, Sayen MR, O'Reilly K, Dillmann WH: Expression of inducible stress protein 70 in rat heart myogenic cells confers protection against simulated ischemia-induced injury. J Clin Invest 1994;93:759-767.

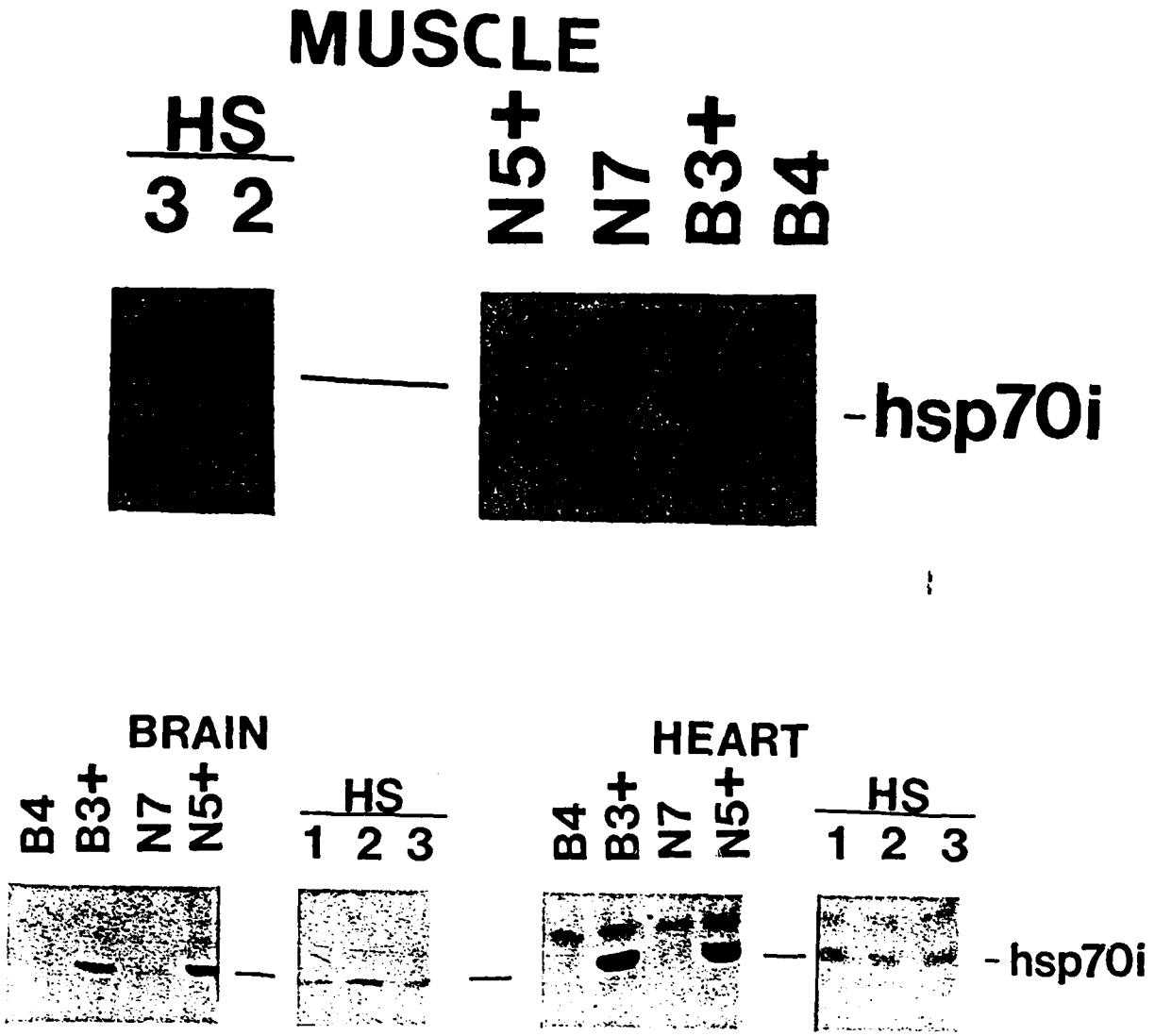

FIGURE 1: Western blot of protein extracts from heart, muscle and brain tissue of negative (Br and N7) and positive (B3 and N5) transgenic mice and non-transgenic mice that were heat shocked (HS 1,2,3) and subsequently recovered for 8-24 hours. Blot was reacted with monoclonal antibody raised against the HSP70 which binds only to the inducible forms of HSP70 (C-92F3A-5, StressGen). Transgenic mice were identified by Southern blot analysis.

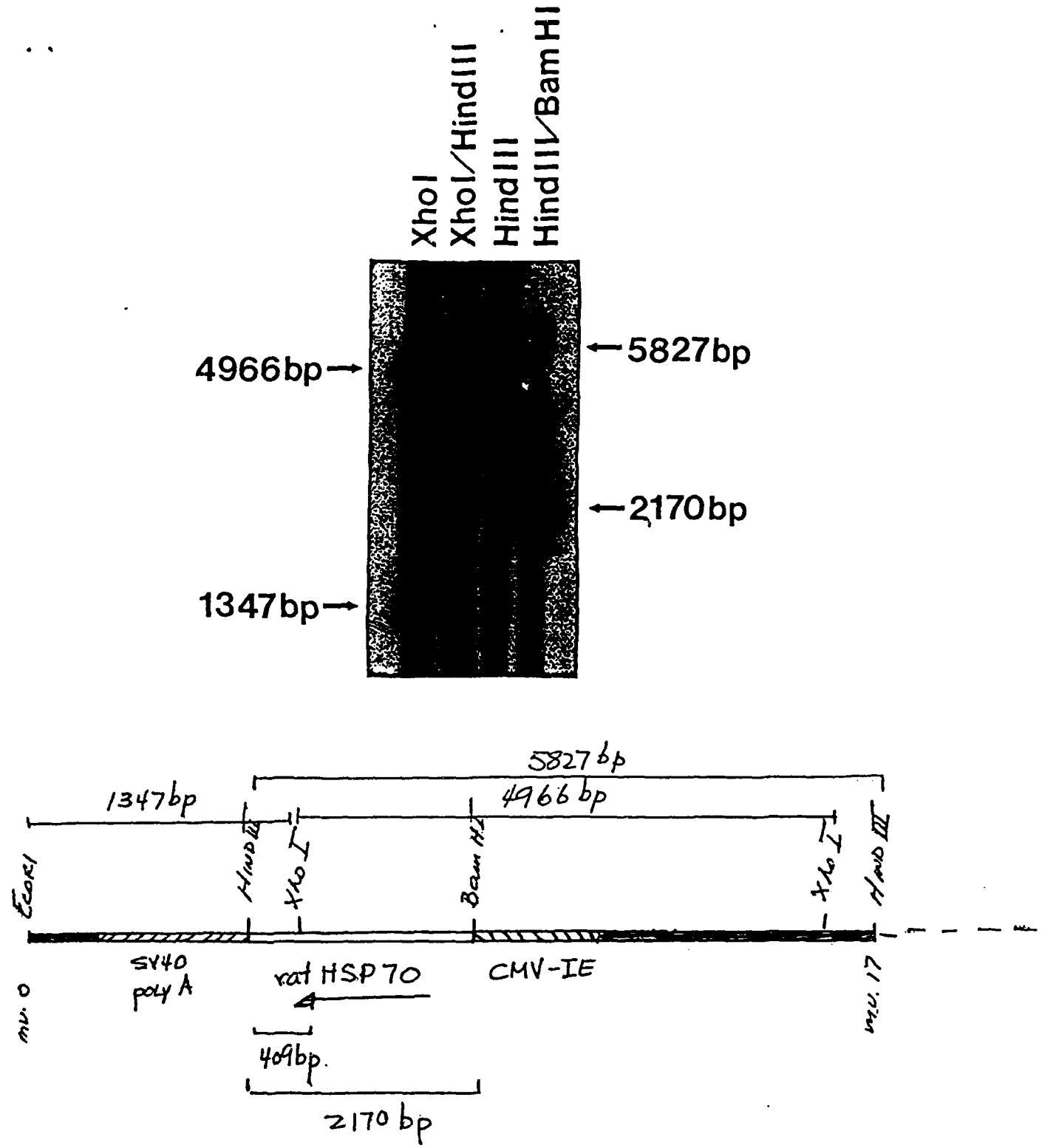

FIGURE 2: Southern blot of adenovirus vector (AdV70) which carries the rat inducible HSP70 coding region. DNA was prepared by the Hirt method from infected 293 cells. AdV70 DNA was digested with several restriction enzymes to confirm the presence of rat HSP70 in the adenovirus vector. The Southern blot was hybridized with the complete coding region of the rat HSP70. Bands in the Southern show the presence of the rat HSP70 in the adenovirus (Hind III/Bam HI digest, 2170 bp band = size of coding region of rHSP70). Band at 1347 bp in lane digested with Xho I shows that orientation of rat HSP70 is in the proper direction.